Influence of hepatic and intestinal efflux transporters and their genetic variants on the pharmacokinetics and pharmacodynamics of raloxifene in osteoporosis treatment

Trdan Lušin, Tina ; Mrhar, Aleš ; Stieger, Bruno ; Kullak-Ublick, Gerd A ; Marc, Janja ; Ostanek, Barbara ; Zavratnik, Andrej ; Kristl, Albin ; Berginc, Katja ; Delić, Katja ; Trontelj, Jurij

Abstract: Raloxifene exhibits a large and unexplained interindividual variability in its pharmacokinetics and pharmacodynamics. The aim of our study was to identify transporters involved in the efflux of raloxifene and its glucuronide metabolites by various in vitro models and by an in vivo study to explore the possible involvement of P-glycoprotein (Pgp), multidrug resistance-associated protein (MRP)1, MRP2, MRP3, and breast cancer resistance protein in the observed high interindividual variability. Experiments with the parallel artificial membrane permeability assay showed the highest passive permeability for raloxifene, followed by raloxifene-6- -glucuronide (M1), raloxifene-4'- -glucuronide (M2), and raloxifene-6,4'-diglucuronide (M3). Caco-2 cell monolayer experiments indicated an interaction of raloxifene with Pgp. The ATPase assay confirmed the raloxifene interaction with Pgp and indicated interactions of all raloxifene species with MRP1, MRP2, MRP3, and breast cancer resistance protein, except for M1, which did not show any interactions with MRP2. Furthermore, the vesicular experiments confirmed the interaction of M2 and M3 with MRP2. Although the in vivo study on osteoporotic postmenopausal women on raloxifene could not confirm a significant influence of ABCB1 and ABCC2 genetic polymorphisms on its pharmacokinetics, a clear trend toward higher total raloxifene concentrations was observed in carriers of at least 1 ABCB1 c.3435T allele. Moreover, the same polymorphism effect was also observed as a significant increase in total hip bone mineral density after 1 year of treatment. The results of our study support the involvement of efflux transporters in disposition of raloxifene and its metabolites and may partially explain the observed raloxifene variability by the influence of the ABCB1 c.3435C>T polymorphism.

DOI: https://doi.org/10.1016/j.trsl.2012.03.002

Influence of hepatic and intestinal efflux transporters and their genetic variants on the pharmacokinetics and pharmacodynamics of raloxifene in osteoporosis treatment

Tina Trdan Lušin[a], Aleš Mrhar[a], Bruno Stieger[b], Gerd A. Kullak-Ublick[b], Janja Marc[c], Barbara Ostanek[c], Andrej Zavratnik[d], Albin Kristl[a], Katja Berginc[a], Katja Delić[c], Jurij Trontelj[a*]

[a]Department of Biopharmacy and Pharmacokinetics, Faculty of Pharmacy, University of Ljubljana, Aškerčeva 7, 1000 Ljubljana, Slovenia

[b]Department of Clinical Pharmacology and Toxicology, University Hospital, Ramistrasse 100, 8091 Zürich, Switzerland

[c]Department of Clinical Biochemistry, Faculty of Pharmacy, University of Ljubljana, Aškerčeva 7, 1000 Ljubljana, Slovenia

[d]Department of Endocrinology and Diabetology, University Medical Centre, Ljubljanska 5, 2000 Maribor, Slovenia

[*]Corresponding author:

Jurij Trontelj, Aškerčeva 7, 1000 Ljubljana; Tel.: +386 1 476 95 47, Fax.: +386 1 425 80 31, E-mail address: jurij.trontelj@ffa.uni-lj.si

Abstract

Raloxifene exhibits quite a large and unexplained interindividual variability in its pharmacokinetics and pharmacodynamics. The aim of our study was to identify transporters involved in the efflux of raloxifene and its glucuronide metabolites by various *in vitro* models and by an *in vivo* study to explore the possible involvement of Pgp, MRP1, MRP2, MRP3, and BCRP in the observed high interindividual variability. Experiments with PAMPA (Parallel Artificial Membrane Permeability Assay) showed the highest passive permeability for raloxifene, followed by raloxifene-6-β-glucuronide (M1), raloxifene-4'-β-glucuronide (M2) and raloxifene-6,4'-diglucuronide (M3). Caco-2 cell monolayer experiments indicated an interaction of raloxifene with Pgp. The ATPase assay confirmed the raloxifene interaction with Pgp, and also indicated interactions of all raloxifene species with MRP1, MRP2, MRP3 and BCRP, except for M1 which did not show any interactions with MRP2. Furthermore, the vesicular experiments confirmed the interaction of M2 and M3 with MRP2. Although the *in vivo* study on osteoporotic postmenopausal women on raloxifene could not confirm a significant influence of *ABCB1* and *ABCC2* genetic polymorphisms on its pharmacokinetics, a clear trend towards higher total raloxifene concentrations was observed in carriers of at least one *ABCB1* c.3435T allele. Moreover, the same polymorphism effect was also observed as a significant increase in total hip bone mineral density after 1 year of treatment. In conclusion, the results of our study support the involvement of efflux transporters in disposition of raloxifene and its metabolites and may partially explain the observed raloxifene variability by the influence of the *ABCB1* c.3435C>T polymorphism.

Keywords: raloxifene, efflux transporters, genetic polymorphisms, pharmacokinetics, pharmacodynamics, translation

Running head and abbreviations

Running head: Efflux transporters and raloxifene

Abbreviations: ABC transporters, ATP-binding cassette transporters; *ABCB1*, ATP-binding cassette transporter B1 gene (Pgp); *ABCC2*, ATP-binding cassette transporter C2 gene (MRP2); BALP, serum bone-specific alkaline phosphatase; BCRP, breast cancer resistance protein; BMD, bone mineral density; BMD-FN, bone mineral density of femoral neck; BMD-HIP, bone mineral density of total hip; BMD-LS, bone mineral density of lumbar spine L1-L4; BSEP, bile salt export pump; BUA, broadband ultrasound attenuation; CTX, serum C-terminal telopeptide fragments of type I collagen; DMSO, dimethylsulfoxide; E17βG , estradiol-17β-glucuronide; FA, formic acid; FLU, fluorescein; FumC, fumitremorgin C; LC-MS/MS, liquid chromatography - tandem mass spectrometry; M1, raloxifene-6-β-glucuronide; M2, raloxifene-4'-β-glucuronide; M3, raloxifene-6,4'-diglucuronide; MRP, multidrug resistance-associated protein; OC, osteocalcin; PAMPA, parallel artificial membrane permeability assay; Pgp, P-glycoprotein; QUI, quantitative ultrasound index; SNP, single nucleotide polymorphism; SOS, heel speed of sound; UGTs, UDP-glucuronosyltransferases.

Introduction

ATP-binding cassette (ABC) transporters work in concert to control the bioavailability of drugs and are thus important determinants of drug-drug interactions, drug efficacy and toxicity. P-glycoprotein (Pgp), multidrug resistance-associated proteins (MRPs), breast cancer resistance protein (BCRP) and bile salt export pump (BSEP) are efflux transporters, which have been well characterized for their ability to transport xenobiotics and endobiotics [1, 2]. Raloxifene, a selective estrogen receptor modulator, is approved worldwide for the prevention and treatment of postmenopausal osteoporosis and for the prevention of breast cancer in postmenopausal women. Raloxifene therapy suppresses the bone turnover to normal premenopausal range, thereby increasing bone mineral density (BMD) in the spine, hip and total body [3, 4], reducing the risk for vertebral fractures [5] but not changing non-vertebral fracture risk [6]. After peroral application, raloxifene undergoes a rapid absorption, an extensive first-pass metabolism and an entero-hepatic cycling. According to the estimations, the fraction of raloxifene absorbed is high, 60 %, but only 2% reaches the systemic circulation in unconjugated form due to the high metabolic turnover during absorption [7]. The rest represents raloxifene conjugated by UDP-glucuronosyltransferases (UGTs) to raloxifene-4'-β-glucuronide (M2), raloxifene-6-β-glucuronide (M1) [7, 8] and raloxifene-6,4'-diglucuronide (M3) [7, 9]. Although the glucuronides show little affinity for the estrogen receptors and for bone tissue, they are important because they can be readily reconverted back into active raloxifene in various organs by β-glucuronidases residing in the liver, lung, spleen, kidney, bone and uterus [10]. Raloxifene and its metabolites are primarily excreted in feces and less than 0.2% and 6% of raloxifene and its metabolites, respectively, are recovered in urine [7]. This fact, coupled with the observation of a major entero-hepatic cycling, suggests an important role of efflux transporters located in the canalicular membrane of hepatocytes and/or in the brush border membrane of intestinal epithelial cells. Previous studies have

shown that raloxifene is probably transported by Pgp [11, 12] while the corresponding glucuronides utilize the MRPs and organic anion transporter(s) (OAT) for their transport [12]. The type of MRP transporter responsible for the transport of raloxifene glucuronides has not been identified yet.

The quite large inter- and intra-individual variability of raloxifene clearance, volume of distribution and thus its pharmacokinetics and/or pharmacodynamics, have been communicated [9] and we believe it could be at least partially connected to the genetic polymorphisms of efflux transporters that are responsible for excretion of raloxifene and its metabolites.

It is well known that genetic polymorphisms present in various drug transporter genes may significantly affect the pharmacokinetics and pharmacodynamics of certain drugs. There are some quite common polymorphisms as for example c.3435C>T in *ABCB1* gene and c.3972C>T in *ABCC2* gene. The *ABCB1* c.3435C>T polymorphism is expected to affect the expression level and function of Pgp [13] but its role in drug pharmacokinetics remains controversial [2]. Nevertheless, it has been published that *ABCB1* c.3435C>T polymorphism influences the pharmacokinetics of paclitaxel [14], cyclosporine [15], phenobarbital [16], nelfinavir [17] and some other drugs. *ABCC2* c.3972C>T is a silent single nucleotide polymorphism (SNP) without any effect on amino acid translation thus the influence of this polymorphism on pharmacokinetics is possibly mediated through a linkage disequilibrium with *C-24T* [18] or with other SNPs in *ABCC2* gene that form different *ABCC2* haplotypes [19]. Our hypothesis was that raloxifene and / or its glucuronides might be substrates for ABC transporters, from which we selected the ones that were most frequently associated with significant impact on pharmacokinetics of various other drugs, namely, Pgp, MRP1, MRP2, MRP3 and BCRP.

The aim of our study was to identify the excretory transporter(s) involved in the efflux of raloxifene and its three glucuronides by different *in vitro* assay systems and by a small *in vivo* study with a special emphasis on the involvement of Pgp, MRP1, MRP2, MRP3 and BCRP. In addition, the influence of Pgp and MRP2 genetic polymorphisms on both pharmacokinetics and pharmacodynamics of raloxifene in postmenopausal osteoporotic women was determined.

1. Materials and methods

2.1. Materials

Raloxifene hydrochloride, fumitremorgin C (Fum C), MK571, verapamil (Ver), haloperidol, fluorescein (FLU), dimethylsulfoxide (DMSO), estradiol-17β-glucuronide (E17βG), formic acid (FA) were from Sigma-Aldrich Chemie (Deisenhofen, Germany). Raloxifene metabolites raloxifene-6-β-glucuronide (M1), raloxifene-4'-β-glucuronide (M2) and raloxifene-6,4'-diglucuronide (M3) were synthesized by incubating raloxifene with Streptomyces sp. ATCC 55043 [20] followed by semipreparative chromatographic purification and lyophilisation. The details of the synthesis and purification are described elsewhere [21]. Purity was checked by HPLC and LC-MS/MS. Stock solutions of raloxifene, M1, M2 and M3 were prepared in DMSO. BD Gentest ™ Pre-coated parallel artificial membrane permeability assays (PAMPA) Plate System was from BD Biosciences (Bedford, USA). PREDEASY™ ATPase Kit was from SOLVO Biotechnology (Szeged, Hungary). ^{3}H-estradiol-17β-glucuronide (^{3}H-E17βG) and taurocholic acid (^{3}H-TCA) were obtained from PerkinElmer Life Sciences (Boston, MA). Caco-2 cells were from American Tissue Culture Collection (ATCC) HTB.37, lot 2463681.

2.2. Permeation experiments across an artificial lipid membrane (PAMPA)

BD Gentest™ Pre-coated PAMPA Plate system was used for the passive permeability determination of raloxifene, M1, M2 and M3. Stock solutions of the compounds were

prepared in DMSO. A donor solution of 20 µM of each compound in 50 mM KH_2PO_4 with 10% methanol was prepared. In each incubation, FLU (10 µM) was used as integrity marker of the membrane. The pH-values of the buffer solutions were adjusted to 6.5 for donor solution and 7.4 for acceptor solution. After incubation of PAMPA plate at room temperature for 5 hours, the samples for LC-MS/MS analysis were diluted four times with water containing 0.5 mg/L haloperidol as an internal standard and analyzed for determination of raloxifene, M1, M2 and M3. For determination of FLU, a fluorescence detector Tecan GENious (λ_{ex} = 485 nm, λ_{em} = 535 nm) was used. 100 µL of a sample was diluted with 100 µL of 0.025 M NaOH before the measurement of FLU. The data were evaluated and the apparent permeabilities (P_{app}) for raloxifene, M1, M2 and M3 were calculated by Eq. (1) and Eq. (2).

$$P_{app} = \frac{-\ln\left[1 - C_A(t)/C_{equilibrium}\right]}{A(1/V_D + 1/V_A)t} \qquad \text{Eq. (1)}$$

$$C_{equilibrium} = \frac{\left[C_D(t) \times V_D + C_A(t) \times V_A\right]}{(V_D + V_A)} \qquad \text{Eq. (2)}$$

In this equations $C_D(t)$ and $C_A(t)$ mean compound concentration in donor and acceptor wells at time t, respectively. V_D is donor well volume (0.3 mL) and V_A is acceptor well volume (0.2 mL), A is filter area (0.3 cm^2) and t is incubation time (in seconds).

2.3. Transport studies with Caco-2 cells

Caco-2 cells were grown on Snapwell Costar culture inserts with a polycarbonate membrane (diameter 12 mm and pore size 0.4 µm). 100,000 cells/filter membrane were used for seeding and the medium was changed every two days. At day 15, transepithelial electrical resistance (TEER) was measured for each filter with Caco-2 cell monolayers. If the TEER values were in the range of 450–750 Ωcm^2, the Caco-2 cell monolayers were used for the subsequent

testing of permeability at day 21. The Caco-2 cells grown on Snapwell Costar culture inserts were carefully rinsed with Ringer buffer and then placed between two compartments of EasyMount™ side-by-side diffusion chambers (Physiologic Instruments, SanDiego, USA). 2.5 mL of bathing solution (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal bovine serum) on each side of the Caco-2 cell monolayer was maintained at 37°C and continuously oxygenated and circulated by bubbling with carbogen (95% O_2, 5% CO_2). After 25 min of preincubation, 0.5 mL of the concentrated solution containing raloxifene was added to the apical side (if studying apical-to-basolateral (A–B) transport) or to the basolateral side (if studying basolateral-to-apical (B–A) transport). The concentrated solution contained 30 µmol/L raloxifene. When evaluating the impact of different ABC efflux transporters on raloxifene transport through Caco-2 cell monolayers, specific inhibitors were added at appropriate concentration to the apical side (Pgp inhibitor verapamil, 100 µmol/L; MRP inhibitor MK571, 25 µmol/L; BCRP inhibitor Fum C, 10 µmol/L). 100 µL of samples were withdrawn from the acceptor side every 20 min up to 100 min, and replaced each time by fresh DMEM containing all necessary ingredients at appropriate concentrations. Caco-2 incubation samples (100 µL) were treated by three volumes of methanol containing 0.5 mg/L of haloperidol as an internal standard and were left on -20°C for 48h, centrifuged at 1300 $\times g$ for 2 hours and the supernatants were subjected for LC-MS/MS analysis. Only those Caco-2 cell monolayers whose TEER values remained constant during the whole experiment were used.

The transepithelial potential difference (PD) and short circuit current (Isc) were measured as shown in Berginc et al. [22]. By monitoring PD, Isc and TEER every 20 min during the experiments, the viability and integrity of Caco-2 cell monolayer were checked.

Data analysis

The apparent permeability coefficient (P_{app}) was calculated according to Eq. (3).

$$P_{app} = \frac{dC \times V}{dt \times C_0 \times A} \quad \text{Eq. (3)}$$

where dc/dt is the change in concentration of the examined substance in the acceptor compartment per unit time under steady state conditions, V is the volume of the acceptor compartment, A the exposed surface area (1.13 cm^2) and c_0 the initial concentration of the examined substance in the donor solution.

When testing the influence of inhibitors of the tested transporters for involvement in raloxifene transport, the efflux ratio (ER) was calculated according to Eq. (4).

$$ER = \frac{P_{app}(B-A)}{P_{app}(A-B)} \quad \text{Eq. (4)}$$

where P_{app}(B - A) and P_{app}(A - B) represent the apparent permeability of raloxifene determined in B - A and A - B directions, respectively. Results in tables are presented as means ± SD of at least three measurements. Data were evaluated statistically using PASW 18.0 for Windows. F-test for testing the equality of variances and, two tailed t-test ($\alpha = 0.05$) were used.

2.4. Membrane-based assays for ABC transporters

2.4.1. ATPase assay

ATPase activities of activated and non-activated transporters (Pgp, MRP1, MRP2, MRP3, BCRP) was measured by using PREDEASY ™ ATPase Kits according to the manufacturer's instructions. In brief, membrane vesicles were diluted with assay mix (50 mM Mops-Tris, 50 mM KCl, 5 mM Na-azide, 2 mM DTT, 0.1 mM EGTA-Tris, 1 mM ouabain in distilled water at pH 7.0) to 0.1 µg/µL and a 40 µL aliquote of this dilution was preincubated with one µL of tested compound diluted in DMSO with or without sodium orthovanadate (1.2 mM) for 10

minutes at 37 °C. Concentration range for tested compounds were 0.39 – 50 µM, 0.32 – 41.4 µM, 0.25 – 31.4 µM and 0.06 – 8 µM for raloxifene, M1, M2 and M3, respectively. After preincubation, the reaction was started by addition of 10 µL of Mg-ATP (0.2 M). The ATPase reaction was stopped after 10 min at 37°C and the concentration of liberated inorganic phosphate was determined colorimetrically by measuring the absorbance at 600 nm. Activation and inhibition experiments were prepared by ATPase assay. For the activation and inhibition experiment in addition to listed compounds 2 mM glutathione was used for MRP1, MRP2 and MRP3. For the inhibition experiment, ATPase assays were performed in the presence of activator substance 40 µM verapamil for Pgp, 10 mM N-ethylmaleimide glutathione for MRP1, 100 µM sulfasalazine for MRP2, 50 µM benzbromarone for MRP3 and 10 µM sulfasalazine for BCRP. Results were calculated using MS Excel 2007.

2.4.2. Membrane vesicular transport studies

BSEP [23] and MRP2 [24] were expressed in Sf9 and Sf21 cells, respectively using standard methodology. Vesicles were isolated from carrier expressing insect cells as described by Gerloff et al. [25]. They were resuspended in 50 mM sucrose, 100 mM KNO_3, 5 mM $Mg(NO_3)_2$, 10 mM HEPES/Tris pH 7.4 and stored in liquid nitrogen until use. Uptake experiment of 2.5 µM taurocholate into BSEP expressing vesicles and 10 µM E17βG into MRP2 expressing vesicles in the presence and absence of 6.25 mM ATP was determined with the rapid-filtration methodology. The vesicular transport was terminated at 0 minutes and 10 minutes and the uptake was calculated first by subtracting the 0 min time point and second by subtracting the uptake in the absence of ATP. Raloxifene, M1, M2 and M3 were tested as inhibitors of E17βG uptake by MRP2 or BSEP at concentrations of 10, 10, 10, 4 µM, respectively.

2.5. Liquid chromatography-tandem mass spectrometry conditions

Caco-2 and PAMPA samples were subjected to LC-MS/MS analysis. The Agilent 1290 Infinity ultra-high pressure liquid chromatographic system (Agilent Techologies, Santa Clara, USA) was coupled to a 6460 Triple Quad Mass Spectrometer (Agilent Techologies, Santa Clara, USA). The chromatographic separation was performed on a Kinetex 50 × 2.1 mm column with 2.6 µm particles with an In-Line filter KrudKatcher Ultra HPLC 0.5 µm and a guard column C18 (2) 4 × 2 mm (Phenomenex, Torrance, USA). The column temperature was 50°C. The mobile phase consisted of 0.1% FA in water (mobile phase A) and 98% acetonitrile (mobile phase B). The flow rate was set at 0.65 mL/min and the separation required a gradient elution. The elution started with 10% of mobile phase B for 0.25 min, and then the elution continued with the following gradient: 10%-20%-30%-50%-50%-10% of mobile phase B in 0.25-0.50-1.00-1.25-1.70-1.75 min, respectively. The chromatographic system was coupled to 6460 Triple Quad Mass Spectrometer with JetStream^{TM} electrospray ionization (Agilent Techologies, Santa Clara, USA) operated in positive mode. Instrument parameters were set as follows: drying gas temperature 275°C, drying gas flow 5 L/min, nebulizer 45 PSI, sheath gas temperature 320°C, sheath gas flow 11 L/min, capillary entrance voltage 4000 V, nozzle voltage 1000V, delta EMV 200V. Both quadrupoles Q1 and Q3 were set at wide mass resolution and dwell time was 50 ms. Instrument control, data acquisition and quantification were performed by MassHunter Workstation software B.03.01 (Agilent Technologies, Torrance, USA). The MRM *m/z* transitions and collision energy characteristics for optimal quantification were 474 → 112, 32 eV for raloxifene, 650 → 474, 28 eV for M1 and M2, 826 → 474, 40 eV for M3 and 376 → 165, 32 eV for haloperidol (internal standard).

2.6. Clinical study

2.6.1. Study design

A total of 57 postmenopausal female patients with osteoporosis were enrolled in the study. The inclusion criteria were: osteoporosis (low BMD, T score <-2.5 SD or presence of verterbral, femoral or radius fracture), 5 years or more of menopause, and age of less than 70. The exclusion criteria were: prior history of venous thromboembolic or malignant disease, serious liver or kidney deficiency, smoking, osteoporosis treatment, lipid lowering or glucocorticoid treatment, estrogen hormone replacement therapy within 6 months prior to the enrolment. The research was carried out according to the principles of the Declaration of Helsinki and was in compliance with local regulatory requirements. A written informed consent was obtained from each individual and the study protocol was approved by the Slovenian National Medical Ethics Committee. The patients were treated for 12 months with 60 mg raloxifene per day and were followed in the University Medical Centre (Maribor, Slovenia). Four patients resigned from the study due to not following the study protocol.

At baseline, blood was drawn for the measurements of bone turnover markers and DNA extraction. All investigations were carried out at 8 a.m. after an overnight fast. After the first visit, the participants started with raloxifene 60 mg, cholecalciferol 400 I.U. and calcium carbonate 1000 mg daily. Patients did not get any dietary advice. Compliance was checked orally by a physician at 3, 6 and 12 months of the therapy. After 12 months, blood was drawn for the measurements of bone turnover markers (serum bone-specific alkaline phosphatase (BALP), serum osteocalcin (OC), serum C-terminal telopeptide fragments of type I collagen (CTX) and concentrations of raloxifene, M1, M2 and M3. After centrifugation, the serum samples were stored at -86°C until analysis. The BMD of total hip (BMD-HIP), femoral neck (BMD-FN) and lumbar spine L1-L4 (BMD-LS,) and three quantitative ultrasound parameters, heel speed of sound (SOS), broadband ultrasound attenuation (BUA) and quantitative ultrasound index (QUI) were measured at baseline and after 12 months of raloxifene therapy.

The statistical power of the study was calculated to be adequate to detect at least 50 %

difference in raloxifene species concentrations among the genotype groups with frequencies of at least 23%, assuming 50% RSD [9]. Additionally, the statistical power was calculated to be sufficient to detect at least 2% difference in delta BMD after 12 months of treatment in different groups with polymorphic genotype frequencies of at least 14% (assuming 50% RSD, α=0.05, 1-β=0.80).

2.6.2. Determination of raloxifene and its metabolites in serum

The method used for determination of M1, M2 and raloxifene had been developed and validated previously in our laboratory and is described in full detail elsewhere [26]. Additionally, the metabolite M3 was monitored. In the present study, the selected reaction monitoring mode (SRM) set at 826→474 (*m/z* transition) was used for the quantification of M3 using the haloperidol as an internal standard. The limit of detection for M3 was 0.039 nM, the limit of quantification was 0.88 nM and the linear range for M3 was from 0.88 nM to 4800 nM. The intra- and inter- day precisions were 2.4 and 1.3%, respectively.

TR (total raloxifene) was calculated as a summation of molar concentrations of raloxifene, M1, M2 and M3.

2.6.3. Determination of pharmacodynamic parameters

BMD-HIP, BMD-FN and BMD-LS were determined by dual energy X-ray absorptiometry (DXA) with a Hologic QDR-2000+ densitometer (Hologic Inc. Waltham, USA).

Quantitative ultrasound (QUS) measurements of the calcaneus were performed on the left heel of all study participants, with the subject in the sitting position, using a Sahara apparatus (Hologic Inc. Waltham, USA). Three QUS parameters of calcaneus were measured: SOS, BUA and QUI. An average of two measurements was calculated.

BALP was assessed by IRMA (Tandem®-R Ostase®; Beckman Coulter). OC was measured using a two-site immunoluminometric Elecsys N-MID Osteocalcin assay (Roche Diagnostics, Mannheim, Germany) [27]. CTX was measured using a two-site immunoluminometric Elecsys Beta CrossLaps assay (Roche Diagnostics, Mannheim Germany) [27].

2.6.4. *ABCB1* and *ABCC2* genotyping

Genomic DNA was isolated from peripheral blood leukocytes by using a FlexiGene DNA kit (Qiagen, Hilden, Germany). Two SNPs in two different genes c.3435C>T (rs1045642) in the *ABCB1* gene and c.3972C>T (rs3740066) in the *ABCC2* gene were genotyped using validated TaqMan Assays (C__7586657_20 and C__11214910_20) from Applied Biosystems in an ABI Prism 7000 sequence detection system, under the conditions recommended by the manufacturer (Applied Biosystems, Foster City, CA, USA). To validate our results, a random selection of 10% of the samples was re-genotyped for each SNP, and the results were found to be reproducible with no discrepancies noted.

2.6.5. Statistical analysis

The Shapiro-Wilk goodness-of-fit test was used to determine the normality of data distribution and the Levene's test was used to test the homogeneity of variances prior to the ANOVA and the t-test. A square root transformation was applied to the concentrations of raloxifene, M1, M2, M3 and TR in order to obtain a normal data distribution. The Bonferroni post hoc test was used to compare the means from genotype groups for each polymorphism. The calculation of percentage change of the pharmacodynamic parameters (PD) during treatment was calculated according to the Eq. (5).

$$\Delta PD\ parameter = \left(\frac{PD\ parameter_{t=0} - PD\ parameter_{t=12\,months}}{PD\ parameter_{0}}\right) \times 100\% \qquad \text{Eq. (5)}$$

To test the influence of genotype on raloxifene species concentration level and on percentage change of pharmacodynamic parameters during treatment, the ANOVA was applied. The Hardy-Weinberg equilibrium was tested for each polymorphism using the χ^2 test. The significance criterion (α) was set at $p < 0.05$. Data analyses were performed by PASW 18.0 software (IBM company, Illinois, Chicago, USA).

3. Results

3.1. Passive permeability measurements

The passive part of raloxifene species permeability was assessed with a PAMPA model. The rank order of the compound permeability through the PAMPA membrane shows that raloxifene has the highest passive permeability, followed by raloxifene monoglucuronides and M3, with the lowest passive permeability (Table 1).

3.2. Transcellular transport assay

Caco-2 cell monolayers were used for the identification of transporters involved in raloxifene transport and for the determination of absorptive raloxifene permeability. The results indicate a decrease in raloxifene efflux ratio, when verapamil (Pgp inhibitor) [22] was applied to the apical side. Fum C (BCRP inhibitor) [28] and MK571 (MRP inhibitor) [29] did not cause any significant changes in the efflux ratio (Table 2). Contrary to the previously published results by Jeong et al. [12], we did not observe any raloxifene glucuronides nor sulfates formation on either donor or acceptor sides.

3.3. ATPase assay

The results of activation and inhibition experiments on transporter ATPases are presented in Tables 3 A and 3 B. It has been previously documented that the modulation of ABC

transporter-specific ATP hydrolysis by a given test compound is a clear sign of its interaction with the protein [30]. The results of activation and inhibition tests for raloxifene, M1, M2 and M3 are shown in Tables 3 A and 3 B. In the activation experiment raloxifene and M3 activated the utilization of ATP by Pgp to promote the transport of these two raloxifene species across the membrane. The utilization of ATP was increased also in the case of M3 when it was incubated with MRP1 membranes. Raloxifene, M1 and M2 increased the utilization of ATP by MRP3. When the tested raloxifene species were incubated with BCRP vesicles there was no change in ATP hydrolysis. In the inhibition experiment an inhibition of activated ATPase activity was observed in all cases except when incubating M1 with MRP2 membranes.

3.4. Vesicular transport (BSEP, MRP2)

Fig. 1 shows the uptake of E17βG as a typical substrate for MRP2 in the absence and presence of raloxifene species as inhibitors. It was observed that M2 and M3, but not M1 and raloxifene significantly inhibited the uptake of E17βG. On the contrary, no inhibition of taurocholate as a typical BSEP substrate was observed in incubation of raloxifene species with BSEP vesicles (data not shown).

3.5. Patient's genotype frequencies

Patient's genotype frequencies for the *ABCB1* c.3435C>T and *ABCC2* c.3972C>T polymorphisms are shown in Table 4. The tested polymorphisms were chosen on the basis of variant allele frequencies that were 46.0%, 32.1% and 9.5% for *ABCB1* c.3435C>T [31], *ABCC2* c.3972C>T [18] and *ABCG2* c. 421C>A [32], respectively. Due to a relatively small number of study participants and low allelic frequency the genotyping for *ABCG2* c. 421C>A

was not performed. All genotype frequencies were found to be in Hardy-Weinberg equilibrium ($p>0.05$).

3.6. Effect of *ABCB1* c.3435C>T and *ABCC2* c.3972C>T on plasma concentration of raloxifene species

The effect of *ABCB1* c.3435C>T and *ABCC2* c.3972C>T polymorphisms on plasma concentration of raloxifene, M1, M2 and M3 was not found to be significant. The *ABCB1* 3435CT and TT variants exhibited a trend toward increased serum concentrations of all tested raloxifene species over homozygous wild-type, but did not reach statistical significance (Table 5).

3.7. Effects of *ABCB1* c.3435C>T and *ABCC2* c.3972C>T on pharmacodynamic parameters

A significant difference in percent change in BMD-HIP after 1 year of raloxifene treatment was observed in subjects according to the *ABCB1* c.3435C>T polymorphism. Patients homozygous for the T allele compared with the heterozygote and the wild-type experienced a higher increase in BMD-HIP after 1 year (Table 6). On the other hand, the changes in BMD-LS and BMD-FN and changes in bone turnover markers were not significant.

4. Discussion

The main goal of our study was to determine which excretory transporters may play a significant role in the disposition of raloxifene and may also be involved in the observed variability in raloxifene exposure and therapeutic effect. Complex *in vitro* and *in vivo* approaches have been utilized to comprehensively describe the excretory transport of raloxifene and its conjugates. We have shown a strong *in vitro* evidence of raloxifene species

interaction with Pgp and MRP transporters which encouraged us to perform an *in vivo* study to evaluate the influence of genetic polymorphisms of these two transporters on both raloxifene pharmacokinetics and pharmacodynamics.

Although raloxifene conjugates do not bind with estrogen receptors with affinity high enough to evoke a pharmacological response they are considered as carriers of active raloxifene [9, 10]. Their blood serum concentration is more than hundred times higher than raloxifene serum concentration (Table 5) and they can be cleaved back to parent raloxifene in many tissues and organs [7, 10]. Therefore, in order to elucidate the complex raloxifene disposition it is vital to understand raloxifene glucuronide membrane transport and its effect on both raloxifene pharmacokinetics and pharmacodynamics.

First, PAMPA was used to predict the passive transcellular permeability of raloxifene, M1, M2 and M3. Secondly, a cell based transport assay for the possible involvement of Pgp, MRP and BCRP was performed on Caco2 cells. Thirdly, two types of membrane-based assays were performed: ATPase assay for Pgp, MRP1, MRP2, MRP3 and BCRP and vesicular transport assay for BSEP and MRP2. Finally, an *in vivo* study on postmenopausal women with osteoporosis was undertaken to test the clinical significance of genetic polymorphisms in genes coding for Pgp and MRP2, which were found to interact either with raloxifene or its glucuronides.

As expected, the PAMPA experiments showed significant differences in passive permeability coefficients of raloxifene species. Raloxifene permeability was the highest, followed by its more hydrophilic metabolites M1, M2 and M3. From these results it is clear that raloxifene conjugates require an active or facilitated transport to efficiently cross the biological membranes. The Caco2 cell line was used for further permeability studies to reveal the possible participation of membrane transporters during the raloxifene absorption phase. The measured raloxifene Caco2 permeability was higher than reported permeability of estradiol

and lower than reported permeability of tamoxifen (7.9 ± 1.1 *vs* 1.2 ± 0.8 *vs* 47.4 ± 21.0 [*10^{-6} cm/s] , respectively) [33, 34]. The obtained efflux ratio for raloxifene (ER) of 11.7 (Table 2) showed that raloxifene is actively excreted from the Caco2 cells, while a significant drop of ER to 6.2 observed in the presence of verapamil (a Pgp inhibitor) suggests that raloxifene excretion from Caco2 cells is largely mediated by Pgp, which is in accordance with findings of Jeong et al. [12] and Chang et al. [11]. On the other hand, no significant changes in efflux ratio were detected with either the MRP or BCRP inhibitors. Caco2 transport experiments with raloxifene conjugates (M1, M2, M3) could not be performed due to the limited amounts of synthesized standards. To investigate the interactions between raloxifene species and individual transporters, the ATP-ase membrane assays and inside-out membrane vesicles were performed, which consumed lesser amounts of metabolite standards. The modulation of ABC transporter-specific ATP hydrolysis by a test compound was taken as a sign of its interaction with the studied transporter. ATP-ase activation suggested the presence of an actively transported compound, while an inhibition of activated transporter (in a separate experiment) indicated a low transport rate or a direct inhibition of the transporter. The results from membrane preparations were complex and rather difficult to interpret. However, it is safe to assume the following conclusions: raloxifene is a Pgp substrate because of the positive results on Caco2 with verapamil and weak activation and strong inhibition of Pgp ATP-ase. Furthermore, M2 and M3 could be MRP2 substrates or inhibitors because of the observed inhibition and activation of MRP2 ATP-ase and significant inhibition of E17βG vesicular uptake. Moreover, there were significant activation and/or inhibition interactions detected between raloxifene species and MRP1 and MRP3 transporters. Therefore it was deemed reasonable to expect a significant influence of the studied transporters on raloxifene disposition. To determine the *in vivo* effects of efflux transporters' genetic polymorphisms, a clinical study on postmenopausal women with osteoporosis was undertaken. Serum

concentrations of raloxifene, M1, M2 and M3 were determined using a validated LC-MS/MS method. Patients were genotyped for *ABCB1* c.3435C>T and *ABCC2* c.3972C>T and it was found that none of the tested polymorphisms significantly influenced the serum concentrations of raloxifene species. However, in the case of *ABCB1* c.3435C>T a trend towards higher concentrations of raloxifene species in patients with at least one polymorphic allele was observed but due to an unexpectedly high interindividual variability it was not significant. The observed polymorphism effect of higher raloxifene species concentrations could be explained by a lower Pgp expression levels in both liver and intestine in carriers of polymorphic alleles leading to a decreased raloxifene species excretion and consequently to their higher serum levels. This explanation is in accordance with the previous results obtained by Hoffmeyer et al. [13] where it was postulated that individuals homozygous for this polymorphism had significantly lower MDR1 expression levels and dysfunction of MDR1. Furthermore, raloxifene is extensively conjugated in both enterocytes and hepatocytes to its metabolites. Consequently, a decreased Pgp activity would also increase raloxifene exposure to the UGTs, therefore this may explain why the concentrations of raloxifene metabolites in serum tended to be higher in patients with polymorphic *MDR1*.

Besides the pharmacokinetic changes, we also evaluated the percent change in pharmacodynamic parameters after 1 year of raloxifene treatment. It was discovered that *ABCB1* c.3435C>T polymorphism significantly influenced the percent change of BMD-HIP after 12 months of raloxifene treatment. Based on the results (Table 6), individuals homozygous for the c.3435T variant experienced a significantly higher increase in BMD-HIP than the wild-type homozygotes. This is in accordance with the observed trend of higher concentrations of raloxifene species in patients with *ABCB1* c.3435TT genotype. To further explore the connection between raloxifene efficacy and safety profile with the presence of the

relatively frequent c. *ABCB1* 3435C>T polymorphism, it would be advisable to conduct a larger prospective cohort study.

In conclusion, we discovered which transporters are most likely involved in the excretion of raloxifene species into bile, which is a very important step in the enterohepatic recirculation of raloxifene and therefore an important determinant for the overall raloxifene exposure. The presented data implicate that at least a part of the observed variability could be explained by the genetic influence of *ABCB1* c.3435C>T polymorphism. In this study, the synergistic use of *in vitro* models combined with a small *in vivo* study has proven to be successful in identification of relevant transporters and confirmation of their *in vivo* effects. The presented multidisciplinary approach of translational research yielded an insight into complex mechanisms of raloxifene disposition and the gained knowledge may thus insure a safer and more effective treatment strategy in the clinical setting.

Acknowledgements

This work was supported by Grants J1-0957 and P3-0298 provided by the Slovenian Research Agency.

All authors have disclosed any financial or personal relationship with organizations that could potentially be perceived as influencing the described research. All authors have read the journal's policy on disclosure of potential conflicts of interest. The authors declare that there are no conflicts of interest.

References

1. Choudhuri S, Klaassen CD. Structure, function, expression, genomic organization, and single nucleotide polymorphisms of human ABCB1 (MDR1), ABCC (MRP), and ABCG2 (BCRP) efflux transporters. Int J Toxicol 2006; 25(4):231-59.

2. Stieger B, Meier PJ. Pharmacogenetics of drug transporters in the enterohepatic circulation. Pharmacogenomics 2011; 12(5):611-31.

3. Delmas PD, Bjarnason NH, Mitlak BH, et al. Effects of raloxifene on bone mineral density, serum cholesterol concentrations, and uterine endometrium in postmenopausal women. N Engl J Med 1997; 337(23):1641-7.

4. Johnston CC, Jr., Bjarnason NH, Cohen FJ, et al. Long-term effects of raloxifene on bone mineral density, bone turnover, and serum lipid levels in early postmenopausal women: three-year data from 2 double-blind, randomized, placebo-controlled trials. Arch Intern Med 2000; 160(22):3444-50.

5. Ettinger B, Black DM, Mitlak BH, et al. Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year

randomized clinical trial. Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators. JAMA 1999; 282(7):637-45.

6. Siris ES, Harris ST, Eastell R, et al. Skeletal effects of raloxifene after 8 years: results from the continuing outcomes relevant to Evista (CORE) study. J Bone Miner Res 2005; 20(9):1514-24.

7. Hochner-Celnikier D. Pharmacokinetics of raloxifene and its clinical application. Eur J Obstet Gynecol Reprod Biol 1999; 85(1):23-9.

8. Jeong EJ, Liu Y, Lin H, Hu M. Species- and disposition model-dependent metabolism of raloxifene in gut and liver: role of UGT1A10. Drug Metab Dispos 2005; 33(6):785-94.

9. U.S.FDA. NDA-020-815 Rockville MD; 1999; Available from: http://www.fda.gov/cder/foi/nda/99/20815S3_Evista.htm (Last accessed: 10February 2009).

10. Dodge JA, Lugar CW, Cho S, et al. Evaluation of the major metabolites of raloxifene as modulators of tissue selectivity. J Steroid Biochem Mol Biol 1997; 61(1-2):97-106.

11. Chang JH, Kochansky CJ, Shou M. The role of P-glycoprotein in the bioactivation of raloxifene. Drug Metab Dispos 2006; 34(12):2073-8.

12. Jeong EJ, Lin H, Hu M. Disposition mechanisms of raloxifene in the human intestinal Caco-2 model. J Pharmacol Exp Ther 2004; 310(1):376-85.

13. Hoffmeyer S, Burk O, von Richter O, et al. Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity in vivo. Proc Natl Acad Sci U S A 2000; 97(7):3473-8.

14. Hemauer SJ, Nanovskaya TN, Abdel-Rahman SZ, Patrikeeva SL, Hankins GD, Ahmed MS. Modulation of human placental P-glycoprotein expression and activity by MDR1 gene polymorphisms. Biochem Pharmacol 2010; 79(6):921-5.

15. Foote CJ, Greer W, Kiberd BA, et al. MDR1 C3435T polymorphisms correlate with cyclosporine levels in de novo renal recipients. Transplant Proc 2006; 38(9):2847-9.

16. Basic S, Hajnsek S, Bozina N, et al. The influence of C3435T polymorphism of ABCB1 gene on penetration of phenobarbital across the blood-brain barrier in patients with generalized epilepsy. Seizure 2008; 17(6):524-30.

17. Saitoh A, Singh KK, Powell CA, et al. An MDR1-3435 variant is associated with higher plasma nelfinavir levels and more rapid virologic response in HIV-1 infected children. AIDS 2005; 19(4):371-80.

18. Naesens M, Kuypers DR, Verbeke K, Vanrenterghem Y. Multidrug resistance protein 2 genetic polymorphisms influence mycophenolic acid exposure in renal allograft recipients. Transplantation 2006; 82(8):1074-84.

19. Laechelt S, Turrini E, Ruehmkorf A, Siegmund W, Cascorbi I, Haenisch S. Impact of ABCC2 haplotypes on transcriptional and posttranscriptional gene regulation and function. Pharmacogenomics J 2011; 11(1):25-34.

20. Briggs B, Baker, PJ ,Belvo, MD, Black, TD, Getman, BG, Kemp, CAJ, Muth, WL, Perun, TJ, Strobel Jr, RJ, Paschal, JW and Zmijewski, MJ. Microbial process for preparation of glucuronides of raloxifene. Journal of Industrial Microbiology & Biotechnology 1999; 23:194–7.

21. Trdan T, Roskar R, Trontelj J, Ravnikar M, Mrhar A. Determination of raloxifene and its glucuronides in human urine by liquid chromatography-tandem mass spectrometry assay. J Chromatogr B Analyt Technol Biomed Life Sci 2011; 879(23):2323-31.

22. Berginc K, Zakelj S, Kristl A. In vitro interactions between aged garlic extract and drugs used for the treatment of cardiovascular and diabetic patients. Eur J Nutr 2010; 49(6):373-84.

23. Noe J, Stieger B, Meier PJ. Functional expression of the canalicular bile salt export pump of human liver. Gastroenterology 2002; 123(5):1659-66.

24. de Waart DR, Paulusma CC, Kunne C, Oude Elferink RP. Multidrug resistance associated protein 2 mediates transport of prostaglandin E2. Liver Int 2006; 26(3):362-8.

25. Gerloff T, Stieger B, Hagenbuch B, et al. The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver. J Biol Chem 1998; 273(16):10046-50.

26. Trontelj J, Bogataj M, Marc J, Mrhar A. Development and validation of a liquid chromatography-tandem mass spectrometry assay for determination of raloxifene and its metabolites in human plasma. J Chromatogr B Analyt Technol Biomed Life Sci 2007; 855(2):220-7.

27. Schmidt-Gayk H, Spanuth E, Kotting J, et al. Performance evaluation of automated assays for beta-CrossLaps, N-MID-Osteocalcin and intact parathyroid hormone (BIOROSE Multicenter Study). Clin Chem Lab Med 2004; 42(1):90-5.

28. Dahan A, Amidon GL. Small intestinal efflux mediated by MRP2 and BCRP shifts sulfasalazine intestinal permeability from high to low, enabling its colonic targeting. Am J Physiol Gastrointest Liver Physiol 2009; 297(2):G371-7.

29. Luders AK, Saborowski R, Bickmeyer U. Inhibition of multidrug/xenobiotic resistance transporter by MK571 improves dye (Fura 2) accumulation in crustacean tissues from lobster, shrimp, and isopod. Comp Biochem Physiol C Toxicol Pharmacol 2009; 150(3):368-71.

30. Hegedus C, Szakacs G, Homolya L, et al. Ins and outs of the ABCG2 multidrug transporter: an update on in vitro functional assays. Adv Drug Deliv Rev 2009; 61(1):47-56.

31. Jeannesson E, Albertini L, Siest G, et al. Determination of ABCB1 polymorphisms and haplotypes frequencies in a French population. Fundam Clin Pharmacol 2007; 21(4):411-8.

32. Keskitalo JE, Zolk O, Fromm MF, Kurkinen KJ, Neuvonen PJ, Niemi M. ABCG2 polymorphism markedly affects the pharmacokinetics of atorvastatin and rosuvastatin. Clin Pharmacol Ther 2009; 86(2):197-203.

33. Aungst BJ, Nguyen NH, Bulgarelli JP, Oates-Lenz K. The influence of donor and reservoir additives on Caco-2 permeability and secretory transport of HIV protease inhibitors and other lipophilic compounds. Pharm Res 2000; 17(10):1175-80.

34. Faassen F, Kelder J, Lenders J, Onderwater R, Vromans H. Physicochemical properties and transport of steroids across Caco-2 cells. Pharm Res 2003; 20(2):177-86.

Legends for figures

Fig. 1: Inhibitory effects of raloxifene, raloxifene-6-β-glucuronide (M1), raloxifene-4'-β-glucuronide (M2), raloxifene-6,4'-diglucuronide (M3) on active uptake of estradiol-17β-glucuronide (E17βG) into MRP2 expressing membrane vesicles. Each column represents the mean (SE) (n=4) and (*) indicates a significant difference from the control ($p < 0.05$).

Influence of hepatic and intestinal efflux transporters and their genetic variants on the pharmacokinetics and pharmacodynamics of raloxifene in osteoporosis treatment

Tina Trdan Lušin[a], Aleš Mrhar[a], Bruno Stieger[b], Gerd A. Kullak-Ublick[b], Janja Marc[c], Barbara Ostanek[c], Andrej Zavratnik[d], Albin Kristl[a], Katja Berginc[a], Katja Delić[c], Jurij Trontelj[a*]

[a]Department of Biopharmacy and Pharmacokinetics, Faculty of Pharmacy, University of Ljubljana, Aškerčeva 7, 1000 Ljubljana, Slovenia

[b]Department of Clinical Pharmacology and Toxicology, University Hospital, Ramistrasse 100, 8091 Zürich, Switzerland

[c]Department of Clinical Biochemistry, Faculty of Pharmacy, University of Ljubljana, Aškerčeva 7, 1000 Ljubljana, Slovenia

[d]Department of Endocrinology and Diabetology, University Medical Centre, Ljubljanska 5, 2000 Maribor, Slovenia

[*]Corresponding author:

Jurij Trontelj, Aškerčeva 7, 1000 Ljubljana; Tel.: +386 1 476 95 47, Fax.: +386 1 425 80 31,

E-mail address: jurij.trontelj@ffa.uni-lj.si

Abstract

Raloxifene exhibits quite a large and unexplained interindividual variability in its pharmacokinetics and pharmacodynamics. The aim of our study was to identify transporters involved in the efflux of raloxifene and its glucuronide metabolites by various *in vitro* models and by an *in vivo* study to explore the possible involvement of Pgp, MRP1, MRP2, MRP3, and BCRP in the observed high interindividual variability. Experiments with PAMPA (Parallel Artificial Membrane Permeability Assay) showed the highest passive permeability for raloxifene, followed by raloxifene-6-β-glucuronide (M1), raloxifene-4'-β-glucuronide (M2) and raloxifene-6,4'-diglucuronide (M3). Caco-2 cell monolayer experiments indicated an interaction of raloxifene with Pgp. The ATPase assay confirmed the raloxifene interaction with Pgp, and also indicated interactions of all raloxifene species with MRP1, MRP2, MRP3 and BCRP, except for M1 which did not show any interactions with MRP2. Furthermore, the vesicular experiments confirmed the interaction of M2 and M3 with MRP2. Although the *in vivo* study on osteoporotic postmenopausal women on raloxifene could not confirm a significant influence of *ABCB1* and *ABCC2* genetic polymorphisms on its pharmacokinetics, a clear trend towards higher total raloxifene concentrations was observed in carriers of at least one *ABCB1* c.3435T allele. Moreover, the same polymorphism effect was also observed as a significant increase in total hip bone mineral density after 1 year of treatment. In conclusion, the results of our study support the involvement of efflux transporters in disposition of raloxifene and its metabolites and may partially explain the observed raloxifene variability by the influence of the *ABCB1* c.3435C>T polymorphism.

Keywords: raloxifene, efflux transporters, genetic polymorphisms, pharmacokinetics, pharmacodynamics, translation

Running head and abbreviations

Running head: Efflux transporters and raloxifene

Abbreviations: ABC transporters, ATP-binding cassette transporters; *ABCB1*, ATP-binding cassette transporter B1 gene (Pgp); *ABCC2*, ATP-binding cassette transporter C2 gene (MRP2); BALP, serum bone-specific alkaline phosphatase; BCRP, breast cancer resistance protein; BMD, bone mineral density; BMD-FN, bone mineral density of femoral neck; BMD-HIP, bone mineral density of total hip; BMD-LS, bone mineral density of lumbar spine L1-L4; BSEP, bile salt export pump; BUA, broadband ultrasound attenuation; CTX, serum C-terminal telopeptide fragments of type I collagen; DMSO, dimethylsulfoxide; E17βG , estradiol-17β-glucuronide; FA, formic acid; FLU, fluorescein; FumC, fumitremorgin C; LC-MS/MS, liquid chromatography - tandem mass spectrometry; M1, raloxifene-6-β-glucuronide; M2, raloxifene-4'-β-glucuronide; M3, raloxifene-6,4'-diglucuronide; MRP, multidrug resistance-associated protein; OC, osteocalcin; PAMPA, parallel artificial membrane permeability assay; Pgp, P-glycoprotein; QUI, quantitative ultrasound index; SNP, single nucleotide polymorphism; SOS, heel speed of sound; UGTs, UDP-glucuronosyltransferases.

Introduction

ATP-binding cassette (ABC) transporters work in concert to control the bioavailability of drugs and are thus important determinants of drug-drug interactions, drug efficacy and toxicity. P-glycoprotein (Pgp), multidrug resistance-associated proteins (MRPs), breast cancer resistance protein (BCRP) and bile salt export pump (BSEP) are efflux transporters, which have been well characterized for their ability to transport xenobiotics and endobiotics [1, 2]. Raloxifene, a selective estrogen receptor modulator, is approved worldwide for the prevention and treatment of postmenopausal osteoporosis and for the prevention of breast cancer in postmenopausal women. Raloxifene therapy suppresses the bone turnover to normal premenopausal range, thereby increasing bone mineral density (BMD) in the spine, hip and total body [3, 4], reducing the risk for vertebral fractures [5] but not changing non-vertebral fracture risk [6]. After peroral application, raloxifene undergoes a rapid absorption, an extensive first-pass metabolism and an entero-hepatic cycling. According to the estimations, the fraction of raloxifene absorbed is high, 60 %, but only 2% reaches the systemic circulation in unconjugated form due to the high metabolic turnover during absorption [7]. The rest represents raloxifene conjugated by UDP-glucuronosyltransferases (UGTs) to raloxifene-4'-β-glucuronide (M2), raloxifene-6-β-glucuronide (M1) [7, 8] and raloxifene-6,4'-diglucuronide (M3) [7, 9]. Although the glucuronides show little affinity for the estrogen receptors and for bone tissue, they are important because they can be readily reconverted back into active raloxifene in various organs by β-glucuronidases residing in the liver, lung, spleen, kidney, bone and uterus [10]. Raloxifene and its metabolites are primarily excreted in feces and less than 0.2% and 6% of raloxifene and its metabolites, respectively, are recovered in urine [7]. This fact, coupled with the observation of a major entero-hepatic cycling, suggests an important role of efflux transporters located in the canalicular membrane of hepatocytes and/or in the brush border membrane of intestinal epithelial cells. Previous studies have

shown that raloxifene is probably transported by Pgp [11, 12] while the corresponding glucuronides utilize the MRPs and organic anion transporter(s) (OAT) for their transport [12]. The type of MRP transporter responsible for the transport of raloxifene glucuronides has not been identified yet.

The quite large inter- and intra-individual variability of raloxifene clearance, volume of distribution and thus its pharmacokinetics and/or pharmacodynamics, have been communicated [9] and we believe it could be at least partially connected to the genetic polymorphisms of efflux transporters that are responsible for excretion of raloxifene and its metabolites.

It is well known that genetic polymorphisms present in various drug transporter genes may significantly affect the pharmacokinetics and pharmacodynamics of certain drugs. There are some quite common polymorphisms as for example c.3435C>T in *ABCB1* gene and c.3972C>T in *ABCC2* gene. The *ABCB1* c.3435C>T polymorphism is expected to affect the expression level and function of Pgp [13] but its role in drug pharmacokinetics remains controversial [2]. Nevertheless, it has been published that *ABCB1* c.3435C>T polymorphism influences the pharmacokinetics of paclitaxel [14], cyclosporine [15], phenobarbital [16], nelfinavir [17] and some other drugs. *ABCC2* c.3972C>T is a silent single nucleotide polymorphism (SNP) without any effect on amino acid translation thus the influence of this polymorphism on pharmacokinetics is possibly mediated through a linkage disequilibrium with *C-24T* [18] or with other SNPs in *ABCC2* gene that form different *ABCC2* haplotypes [19]. Our hypothesis was that raloxifene and / or its glucuronides might be substrates for ABC transporters, from which we selected the ones that were most frequently associated with significant impact on pharmacokinetics of various other drugs, namely, Pgp, MRP1, MRP2, MRP3 and BCRP.

The aim of our study was to identify the excretory transporter(s) involved in the efflux of raloxifene and its three glucuronides by different *in vitro* assay systems and by a small *in vivo* study with a special emphasis on the involvement of Pgp, MRP1, MRP2, MRP3 and BCRP. In addition, the influence of Pgp and MRP2 genetic polymorphisms on both pharmacokinetics and pharmacodynamics of raloxifene in postmenopausal osteoporotic women was determined.

1. Materials and methods

2.1. Materials

Raloxifene hydrochloride, fumitremorgin C (Fum C), MK571, verapamil (Ver), haloperidol, fluorescein (FLU), dimethylsulfoxide (DMSO), estradiol-17β-glucuronide (E17βG), formic acid (FA) were from Sigma-Aldrich Chemie (Deisenhofen, Germany). Raloxifene metabolites raloxifene-6-β-glucuronide (M1), raloxifene-4'-β-glucuronide (M2) and raloxifene-6,4'-diglucuronide (M3) were synthesized by incubating raloxifene with Streptomyces sp. ATCC 55043 [20] followed by semipreparative chromatographic purification and lyophilisation. The details of the synthesis and purification are described elsewhere [21]. Purity was checked by HPLC and LC-MS/MS. Stock solutions of raloxifene, M1, M2 and M3 were prepared in DMSO. BD Gentest ™ Pre-coated parallel artificial membrane permeability assays (PAMPA) Plate System was from BD Biosciences (Bedford, USA). PREDEASY™ ATPase Kit was from SOLVO Biotechnology (Szeged, Hungary). ^{3}H-estradiol-17β-glucuronide (^{3}H-E17βG) and taurocholic acid (^{3}H-TCA) were obtained from PerkinElmer Life Sciences (Boston, MA). Caco-2 cells were from American Tissue Culture Collection (ATCC) HTB.37, lot 2463681.

2.2. Permeation experiments across an artificial lipid membrane (PAMPA)

BD Gentest™ Pre-coated PAMPA Plate system was used for the passive permeability determination of raloxifene, M1, M2 and M3. Stock solutions of the compounds were

prepared in DMSO. A donor solution of 20 µM of each compound in 50 mM KH_2PO_4 with 10% methanol was prepared. In each incubation, FLU (10 µM) was used as integrity marker of the membrane. The pH-values of the buffer solutions were adjusted to 6.5 for donor solution and 7.4 for acceptor solution. After incubation of PAMPA plate at room temperature for 5 hours, the samples for LC-MS/MS analysis were diluted four times with water containing 0.5 mg/L haloperidol as an internal standard and analyzed for determination of raloxifene, M1, M2 and M3. For determination of FLU, a fluorescence detector Tecan GENious (λ_{ex} = 485 nm, λ_{em} = 535 nm) was used. 100 µL of a sample was diluted with 100 µL of 0.025 M NaOH before the measurement of FLU. The data were evaluated and the apparent permeabilities (P_{app}) for raloxifene, M1, M2 and M3 were calculated by Eq. (1) and Eq. (2).

$$P_{app} = \frac{-\ln\left[1 - C_A(t)/C_{equilibrium}\right]}{A(1/V_D + 1/V_A)t} \qquad \text{Eq. (1)}$$

$$C_{equilibrium} = \frac{\left[C_D(t) \times V_D + C_A(t) \times V_A\right]}{(V_D + V_A)} \qquad \text{Eq. (2)}$$

In this equations $C_D(t)$ and $C_A(t)$ mean compound concentration in donor and acceptor wells at time t, respectively. V_D is donor well volume (0.3 mL) and V_A is acceptor well volume (0.2 mL), A is filter area (0.3 cm^2) and t is incubation time (in seconds).

2.3. Transport studies with Caco-2 cells

Caco-2 cells were grown on Snapwell Costar culture inserts with a polycarbonate membrane (diameter 12 mm and pore size 0.4 µm). 100,000 cells/filter membrane were used for seeding and the medium was changed every two days. At day 15, transepithelial electrical resistance (TEER) was measured for each filter with Caco-2 cell monolayers. If the TEER values were in the range of 450–750 Ωcm^2, the Caco-2 cell monolayers were used for the subsequent

testing of permeability at day 21. The Caco-2 cells grown on Snapwell Costar culture inserts were carefully rinsed with Ringer buffer and then placed between two compartments of EasyMount™ side-by-side diffusion chambers (Physiologic Instruments, SanDiego, USA). 2.5 mL of bathing solution (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal bovine serum) on each side of the Caco-2 cell monolayer was maintained at 37°C and continuously oxygenated and circulated by bubbling with carbogen (95% O_2, 5% CO_2). After 25 min of preincubation, 0.5 mL of the concentrated solution containing raloxifene was added to the apical side (if studying apical-to-basolateral (A–B) transport) or to the basolateral side (if studying basolateral-to-apical (B–A) transport). The concentrated solution contained 30 µmol/L raloxifene. When evaluating the impact of different ABC efflux transporters on raloxifene transport through Caco-2 cell monolayers, specific inhibitors were added at appropriate concentration to the apical side (Pgp inhibitor verapamil, 100 µmol/L; MRP inhibitor MK571, 25 µmol/L; BCRP inhibitor Fum C, 10 µmol/L). 100 µL of samples were withdrawn from the acceptor side every 20 min up to 100 min, and replaced each time by fresh DMEM containing all necessary ingredients at appropriate concentrations. Caco-2 incubation samples (100 µL) were treated by three volumes of methanol containing 0.5 mg/L of haloperidol as an internal standard and were left on -20°C for 48h, centrifuged at 1300 $\times g$ for 2 hours and the supernatants were subjected for LC-MS/MS analysis. Only those Caco-2 cell monolayers whose TEER values remained constant during the whole experiment were used.

The transepithelial potential difference (PD) and short circuit current (Isc) were measured as shown in Berginc et al. [22]. By monitoring PD, Isc and TEER every 20 min during the experiments, the viability and integrity of Caco-2 cell monolayer were checked.

Data analysis

The apparent permeability coefficient (P_{app}) was calculated according to Eq. (3).

$$P_{app} = \frac{dC \times V}{dt \times C_0 \times A} \quad \text{Eq. (3)}$$

where dc/dt is the change in concentration of the examined substance in the acceptor compartment per unit time under steady state conditions, V is the volume of the acceptor compartment, A the exposed surface area (1.13 cm^2) and c_0 the initial concentration of the examined substance in the donor solution.

When testing the influence of inhibitors of the tested transporters for involvement in raloxifene transport, the efflux ratio (ER) was calculated according to Eq. (4).

$$ER = \frac{P_{app}(B-A)}{P_{app}(A-B)} \quad \text{Eq. (4)}$$

where P_{app}(B - A) and P_{app}(A - B) represent the apparent permeability of raloxifene determined in B - A and A - B directions, respectively. Results in tables are presented as means ± SD of at least three measurements. Data were evaluated statistically using PASW 18.0 for Windows. F-test for testing the equality of variances and, two tailed t-test ($\alpha = 0.05$) were used.

2.4. Membrane-based assays for ABC transporters

2.4.1. ATPase assay

ATPase activities of activated and non-activated transporters (Pgp, MRP1, MRP2, MRP3, BCRP) was measured by using PREDEASY ™ ATPase Kits according to the manufacturer's instructions. In brief, membrane vesicles were diluted with assay mix (50 mM Mops-Tris, 50 mM KCl, 5 mM Na-azide, 2 mM DTT, 0.1 mM EGTA-Tris, 1 mM ouabain in distilled water at pH 7.0) to 0.1 µg/µL and a 40 µL aliquote of this dilution was preincubated with one µL of tested compound diluted in DMSO with or without sodium orthovanadate (1.2 mM) for 10

minutes at 37 °C. Concentration range for tested compounds were 0.39 – 50 µM, 0.32 – 41.4 µM, 0.25 – 31.4 µM and 0.06 – 8 µM for raloxifene, M1, M2 and M3, respectively. After preincubation, the reaction was started by addition of 10 µL of Mg-ATP (0.2 M). The ATPase reaction was stopped after 10 min at 37°C and the concentration of liberated inorganic phosphate was determined colorimetrically by measuring the absorbance at 600 nm. Activation and inhibition experiments were prepared by ATPase assay. For the activation and inhibition experiment in addition to listed compounds 2 mM glutathione was used for MRP1, MRP2 and MRP3. For the inhibition experiment, ATPase assays were performed in the presence of activator substance 40 µM verapamil for Pgp, 10 mM N-ethylmaleimide glutathione for MRP1, 100 µM sulfasalazine for MRP2, 50 µM benzbromarone for MRP3 and 10 µM sulfasalazine for BCRP. Results were calculated using MS Excel 2007.

2.4.2. Membrane vesicular transport studies

BSEP [23] and MRP2 [24] were expressed in Sf9 and Sf21 cells, respectively using standard methodology. Vesicles were isolated from carrier expressing insect cells as described by Gerloff et al. [25]. They were resuspended in 50 mM sucrose, 100 mM KNO_3, 5 mM $Mg(NO_3)_2$, 10 mM HEPES/Tris pH 7.4 and stored in liquid nitrogen until use. Uptake experiment of 2.5 µM taurocholate into BSEP expressing vesicles and 10 µM E17βG into MRP2 expressing vesicles in the presence and absence of 6.25 mM ATP was determined with the rapid-filtration methodology. The vesicular transport was terminated at 0 minutes and 10 minutes and the uptake was calculated first by subtracting the 0 min time point and second by subtracting the uptake in the absence of ATP. Raloxifene, M1, M2 and M3 were tested as inhibitors of E17βG uptake by MRP2 or BSEP at concentrations of 10, 10, 10, 4 µM, respectively.

2.5. Liquid chromatography-tandem mass spectrometry conditions

Caco-2 and PAMPA samples were subjected to LC-MS/MS analysis. The Agilent 1290 Infinity ultra-high pressure liquid chromatographic system (Agilent Techologies, Santa Clara, USA) was coupled to a 6460 Triple Quad Mass Spectrometer (Agilent Techologies, Santa Clara, USA). The chromatographic separation was performed on a Kinetex 50 × 2.1 mm column with 2.6 µm particles with an In-Line filter KrudKatcher Ultra HPLC 0.5 µm and a guard column C18 (2) 4 × 2 mm (Phenomenex, Torrance, USA). The column temperature was 50°C. The mobile phase consisted of 0.1% FA in water (mobile phase A) and 98% acetonitrile (mobile phase B). The flow rate was set at 0.65 mL/min and the separation required a gradient elution. The elution started with 10% of mobile phase B for 0.25 min, and then the elution continued with the following gradient: 10%-20%-30%-50%-50%-10% of mobile phase B in 0.25-0.50-1.00-1.25-1.70-1.75 min, respectively. The chromatographic system was coupled to 6460 Triple Quad Mass Spectrometer with JetStream^{TM} electrospray ionization (Agilent Techologies, Santa Clara, USA) operated in positive mode. Instrument parameters were set as follows: drying gas temperature 275°C, drying gas flow 5 L/min, nebulizer 45 PSI, sheath gas temperature 320°C, sheath gas flow 11 L/min, capillary entrance voltage 4000 V, nozzle voltage 1000V, delta EMV 200V. Both quadrupoles Q1 and Q3 were set at wide mass resolution and dwell time was 50 ms. Instrument control, data acquisition and quantification were performed by MassHunter Workstation software B.03.01 (Agilent Technologies, Torrance, USA). The MRM *m/z* transitions and collision energy characteristics for optimal quantification were 474 → 112, 32 eV for raloxifene, 650 → 474, 28 eV for M1 and M2, 826 → 474, 40 eV for M3 and 376 → 165, 32 eV for haloperidol (internal standard).

2.6. Clinical study

2.6.1. Study design

A total of 57 postmenopausal female patients with osteoporosis were enrolled in the study. The inclusion criteria were: osteoporosis (low BMD, T score <-2.5 SD or presence of verterbral, femoral or radius fracture), 5 years or more of menopause, and age of less than 70. The exclusion criteria were: prior history of venous thromboembolic or malignant disease, serious liver or kidney deficiency, smoking, osteoporosis treatment, lipid lowering or glucocorticoid treatment, estrogen hormone replacement therapy within 6 months prior to the enrolment. The research was carried out according to the principles of the Declaration of Helsinki and was in compliance with local regulatory requirements. A written informed consent was obtained from each individual and the study protocol was approved by the Slovenian National Medical Ethics Committee. The patients were treated for 12 months with 60 mg raloxifene per day and were followed in the University Medical Centre (Maribor, Slovenia). Four patients resigned from the study due to not following the study protocol.

At baseline, blood was drawn for the measurements of bone turnover markers and DNA extraction. All investigations were carried out at 8 a.m. after an overnight fast. After the first visit, the participants started with raloxifene 60 mg, cholecalciferol 400 I.U. and calcium carbonate 1000 mg daily. Patients did not get any dietary advice. Compliance was checked orally by a physician at 3, 6 and 12 months of the therapy. After 12 months, blood was drawn for the measurements of bone turnover markers (serum bone-specific alkaline phosphatase (BALP), serum osteocalcin (OC), serum C-terminal telopeptide fragments of type I collagen (CTX) and concentrations of raloxifene, M1, M2 and M3. After centrifugation, the serum samples were stored at -86°C until analysis. The BMD of total hip (BMD-HIP), femoral neck (BMD-FN) and lumbar spine L1-L4 (BMD-LS,) and three quantitative ultrasound parameters, heel speed of sound (SOS), broadband ultrasound attenuation (BUA) and quantitative ultrasound index (QUI) were measured at baseline and after 12 months of raloxifene therapy. The statistical power of the study was calculated to be adequate to detect at least 50 %

difference in raloxifene species concentrations among the genotype groups with frequencies of at least 23%, assuming 50% RSD [9]. Additionally, the statistical power was calculated to be sufficient to detect at least 2% difference in delta BMD after 12 months of treatment in different groups with polymorphic genotype frequencies of at least 14% (assuming 50% RSD, α=0.05, 1-β=0.80).

2.6.2. Determination of raloxifene and its metabolites in serum

The method used for determination of M1, M2 and raloxifene had been developed and validated previously in our laboratory and is described in full detail elsewhere [26]. Additionally, the metabolite M3 was monitored. In the present study, the selected reaction monitoring mode (SRM) set at 826→474 (*m/z* transition) was used for the quantification of M3 using the haloperidol as an internal standard. The limit of detection for M3 was 0.039 nM, the limit of quantification was 0.88 nM and the linear range for M3 was from 0.88 nM to 4800 nM. The intra- and inter- day precisions were 2.4 and 1.3%, respectively.

TR (total raloxifene) was calculated as a summation of molar concentrations of raloxifene, M1, M2 and M3.

2.6.3. Determination of pharmacodynamic parameters

BMD-HIP, BMD-FN and BMD-LS were determined by dual energy X-ray absorptiometry (DXA) with a Hologic QDR-2000+ densitometer (Hologic Inc. Waltham, USA).

Quantitative ultrasound (QUS) measurements of the calcaneus were performed on the left heel of all study participants, with the subject in the sitting position, using a Sahara apparatus (Hologic Inc. Waltham, USA). Three QUS parameters of calcaneus were measured: SOS, BUA and QUI. An average of two measurements was calculated.

BALP was assessed by IRMA (Tandem®-R Ostase®; Beckman Coulter). OC was measured using a two-site immunoluminometric Elecsys N-MID Osteocalcin assay (Roche Diagnostics, Mannheim, Germany) [27]. CTX was measured using a two-site immunoluminometric Elecsys Beta CrossLaps assay (Roche Diagnostics, Mannheim Germany) [27].

2.6.4. *ABCB1* and *ABCC2* genotyping

Genomic DNA was isolated from peripheral blood leukocytes by using a FlexiGene DNA kit (Qiagen, Hilden, Germany). Two SNPs in two different genes c.3435C>T (rs1045642) in the *ABCB1* gene and c.3972C>T (rs3740066) in the *ABCC2* gene were genotyped using validated TaqMan Assays (C__7586657_20 and C__11214910_20) from Applied Biosystems in an ABI Prism 7000 sequence detection system, under the conditions recommended by the manufacturer (Applied Biosystems, Foster City, CA, USA). To validate our results, a random selection of 10% of the samples was re-genotyped for each SNP, and the results were found to be reproducible with no discrepancies noted.

2.6.5. Statistical analysis

The Shapiro-Wilk goodness-of-fit test was used to determine the normality of data distribution and the Levene's test was used to test the homogeneity of variances prior to the ANOVA and the t-test. A square root transformation was applied to the concentrations of raloxifene, M1, M2, M3 and TR in order to obtain a normal data distribution. The Bonferroni post hoc test was used to compare the means from genotype groups for each polymorphism. The calculation of percentage change of the pharmacodynamic parameters (PD) during treatment was calculated according to the Eq. (5).

$$\Delta PD\ parameter = \left(\frac{PD\ parameter_{t=0} - PD\ parameter_{t=12\,months}}{PD\ parameter_{0}}\right) \times 100\% \qquad \text{Eq. (5)}$$

To test the influence of genotype on raloxifene species concentration level and on percentage change of pharmacodynamic parameters during treatment, the ANOVA was applied. The Hardy-Weinberg equilibrium was tested for each polymorphism using the χ^2 test. The significance criterion (α) was set at $p < 0.05$. Data analyses were performed by PASW 18.0 software (IBM company, Illinois, Chicago, USA).

3. Results

3.1. Passive permeability measurements

The passive part of raloxifene species permeability was assessed with a PAMPA model. The rank order of the compound permeability through the PAMPA membrane shows that raloxifene has the highest passive permeability, followed by raloxifene monoglucuronides and M3, with the lowest passive permeability (Table 1).

3.2. Transcellular transport assay

Caco-2 cell monolayers were used for the identification of transporters involved in raloxifene transport and for the determination of absorptive raloxifene permeability. The results indicate a decrease in raloxifene efflux ratio, when verapamil (Pgp inhibitor) [22] was applied to the apical side. Fum C (BCRP inhibitor) [28] and MK571 (MRP inhibitor) [29] did not cause any significant changes in the efflux ratio (Table 2). Contrary to the previously published results by Jeong et al. [12], we did not observe any raloxifene glucuronides nor sulfates formation on either donor or acceptor sides.

3.3. ATPase assay

The results of activation and inhibition experiments on transporter ATPases are presented in Tables 3 A and 3 B. It has been previously documented that the modulation of ABC

transporter-specific ATP hydrolysis by a given test compound is a clear sign of its interaction with the protein [30]. The results of activation and inhibition tests for raloxifene, M1, M2 and M3 are shown in Tables 3 A and 3 B. In the activation experiment raloxifene and M3 activated the utilization of ATP by Pgp to promote the transport of these two raloxifene species across the membrane. The utilization of ATP was increased also in the case of M3 when it was incubated with MRP1 membranes. Raloxifene, M1 and M2 increased the utilization of ATP by MRP3. When the tested raloxifene species were incubated with BCRP vesicles there was no change in ATP hydrolysis. In the inhibition experiment an inhibition of activated ATPase activity was observed in all cases except when incubating M1 with MRP2 membranes.

3.4. Vesicular transport (BSEP, MRP2)

Fig. 1 shows the uptake of E17βG as a typical substrate for MRP2 in the absence and presence of raloxifene species as inhibitors. It was observed that M2 and M3, but not M1 and raloxifene significantly inhibited the uptake of E17βG. On the contrary, no inhibition of taurocholate as a typical BSEP substrate was observed in incubation of raloxifene species with BSEP vesicles (data not shown).

3.5. Patient's genotype frequencies

Patient's genotype frequencies for the *ABCB1* c.3435C>T and *ABCC2* c.3972C>T polymorphisms are shown in Table 4. The tested polymorphisms were chosen on the basis of variant allele frequencies that were 46.0%, 32.1% and 9.5% for *ABCB1* c.3435C>T [31], *ABCC2* c.3972C>T [18] and *ABCG2* c. 421C>A [32], respectively. Due to a relatively small number of study participants and low allelic frequency the genotyping for *ABCG2* c. 421C>A

was not performed. All genotype frequencies were found to be in Hardy-Weinberg equilibrium ($p>0.05$).

3.6. Effect of *ABCB1* c.3435C>T and *ABCC2* c.3972C>T on plasma concentration of raloxifene species

The effect of *ABCB1* c.3435C>T and *ABCC2* c.3972C>T polymorphisms on plasma concentration of raloxifene, M1, M2 and M3 was not found to be significant. The *ABCB1* 3435CT and TT variants exhibited a trend toward increased serum concentrations of all tested raloxifene species over homozygous wild-type, but did not reach statistical significance (Table 5).

3.7. Effects of *ABCB1* c.3435C>T and *ABCC2* c.3972C>T on pharmacodynamic parameters

A significant difference in percent change in BMD-HIP after 1 year of raloxifene treatment was observed in subjects according to the *ABCB1* c.3435C>T polymorphism. Patients homozygous for the T allele compared with the heterozygote and the wild-type experienced a higher increase in BMD-HIP after 1 year (Table 6). On the other hand, the changes in BMD-LS and BMD-FN and changes in bone turnover markers were not significant.

4. Discussion

The main goal of our study was to determine which excretory transporters may play a significant role in the disposition of raloxifene and may also be involved in the observed variability in raloxifene exposure and therapeutic effect. Complex *in vitro* and *in vivo* approaches have been utilized to comprehensively describe the excretory transport of raloxifene and its conjugates. We have shown a strong *in vitro* evidence of raloxifene species

interaction with Pgp and MRP transporters which encouraged us to perform an *in vivo* study to evaluate the influence of genetic polymorphisms of these two transporters on both raloxifene pharmacokinetics and pharmacodynamics.

Although raloxifene conjugates do not bind with estrogen receptors with affinity high enough to evoke a pharmacological response they are considered as carriers of active raloxifene [9, 10]. Their blood serum concentration is more than hundred times higher than raloxifene serum concentration (Table 5) and they can be cleaved back to parent raloxifene in many tissues and organs [7, 10]. Therefore, in order to elucidate the complex raloxifene disposition it is vital to understand raloxifene glucuronide membrane transport and its effect on both raloxifene pharmacokinetics and pharmacodynamics.

First, PAMPA was used to predict the passive transcellular permeability of raloxifene, M1, M2 and M3. Secondly, a cell based transport assay for the possible involvement of Pgp, MRP and BCRP was performed on Caco2 cells. Thirdly, two types of membrane-based assays were performed: ATPase assay for Pgp, MRP1, MRP2, MRP3 and BCRP and vesicular transport assay for BSEP and MRP2. Finally, an *in vivo* study on postmenopausal women with osteoporosis was undertaken to test the clinical significance of genetic polymorphisms in genes coding for Pgp and MRP2, which were found to interact either with raloxifene or its glucuronides.

As expected, the PAMPA experiments showed significant differences in passive permeability coefficients of raloxifene species. Raloxifene permeability was the highest, followed by its more hydrophilic metabolites M1, M2 and M3. From these results it is clear that raloxifene conjugates require an active or facilitated transport to efficiently cross the biological membranes. The Caco2 cell line was used for further permeability studies to reveal the possible participation of membrane transporters during the raloxifene absorption phase. The measured raloxifene Caco2 permeability was higher than reported permeability of estradiol

and lower than reported permeability of tamoxifen (7.9 ± 1.1 *vs* 1.2 ± 0.8 *vs* 47.4 ± 21.0 [*10^{-6} cm/s] , respectively) [33, 34]. The obtained efflux ratio for raloxifene (ER) of 11.7 (Table 2) showed that raloxifene is actively excreted from the Caco2 cells, while a significant drop of ER to 6.2 observed in the presence of verapamil (a Pgp inhibitor) suggests that raloxifene excretion from Caco2 cells is largely mediated by Pgp, which is in accordance with findings of Jeong et al. [12] and Chang et al. [11]. On the other hand, no significant changes in efflux ratio were detected with either the MRP or BCRP inhibitors. Caco2 transport experiments with raloxifene conjugates (M1, M2, M3) could not be performed due to the limited amounts of synthesized standards. To investigate the interactions between raloxifene species and individual transporters, the ATP-ase membrane assays and inside-out membrane vesicles were performed, which consumed lesser amounts of metabolite standards. The modulation of ABC transporter-specific ATP hydrolysis by a test compound was taken as a sign of its interaction with the studied transporter. ATP-ase activation suggested the presence of an actively transported compound, while an inhibition of activated transporter (in a separate experiment) indicated a low transport rate or a direct inhibition of the transporter. The results from membrane preparations were complex and rather difficult to interpret. However, it is safe to assume the following conclusions: raloxifene is a Pgp substrate because of the positive results on Caco2 with verapamil and weak activation and strong inhibition of Pgp ATP-ase. Furthermore, M2 and M3 could be MRP2 substrates or inhibitors because of the observed inhibition and activation of MRP2 ATP-ase and significant inhibition of E17βG vesicular uptake. Moreover, there were significant activation and/or inhibition interactions detected between raloxifene species and MRP1 and MRP3 transporters. Therefore it was deemed reasonable to expect a significant influence of the studied transporters on raloxifene disposition. To determine the *in vivo* effects of efflux transporters' genetic polymorphisms, a clinical study on postmenopausal women with osteoporosis was undertaken. Serum

concentrations of raloxifene, M1, M2 and M3 were determined using a validated LC-MS/MS method. Patients were genotyped for *ABCB1* c.3435C>T and *ABCC2* c.3972C>T and it was found that none of the tested polymorphisms significantly influenced the serum concentrations of raloxifene species. However, in the case of *ABCB1* c.3435C>T a trend towards higher concentrations of raloxifene species in patients with at least one polymorphic allele was observed but due to an unexpectedly high interindividual variability it was not significant. The observed polymorphism effect of higher raloxifene species concentrations could be explained by a lower Pgp expression levels in both liver and intestine in carriers of polymorphic alleles leading to a decreased raloxifene species excretion and consequently to their higher serum levels. This explanation is in accordance with the previous results obtained by Hoffmeyer et al. [13] where it was postulated that individuals homozygous for this polymorphism had significantly lower MDR1 expression levels and dysfunction of MDR1. Furthermore, raloxifene is extensively conjugated in both enterocytes and hepatocytes to its metabolites. Consequently, a decreased Pgp activity would also increase raloxifene exposure to the UGTs, therefore this may explain why the concentrations of raloxifene metabolites in serum tended to be higher in patients with polymorphic *MDR1*.

Besides the pharmacokinetic changes, we also evaluated the percent change in pharmacodynamic parameters after 1 year of raloxifene treatment. It was discovered that *ABCB1* c.3435C>T polymorphism significantly influenced the percent change of BMD-HIP after 12 months of raloxifene treatment. Based on the results (Table 6), individuals homozygous for the c.3435T variant experienced a significantly higher increase in BMD-HIP than the wild-type homozygotes. This is in accordance with the observed trend of higher concentrations of raloxifene species in patients with *ABCB1* c.3435TT genotype. To further explore the connection between raloxifene efficacy and safety profile with the presence of the

relatively frequent c. *ABCB1* 3435C>T polymorphism, it would be advisable to conduct a larger prospective cohort study.

In conclusion, we discovered which transporters are most likely involved in the excretion of raloxifene species into bile, which is a very important step in the enterohepatic recirculation of raloxifene and therefore an important determinant for the overall raloxifene exposure. The presented data implicate that at least a part of the observed variability could be explained by the genetic influence of *ABCB1* c.3435C>T polymorphism. In this study, the synergistic use of *in vitro* models combined with a small *in vivo* study has proven to be successful in identification of relevant transporters and confirmation of their *in vivo* effects. The presented multidisciplinary approach of translational research yielded an insight into complex mechanisms of raloxifene disposition and the gained knowledge may thus insure a safer and more effective treatment strategy in the clinical setting.

Acknowledgements

This work was supported by Grants J1-0957 and P3-0298 provided by the Slovenian Research Agency.

All authors have disclosed any financial or personal relationship with organizations that could potentially be perceived as influencing the described research. All authors have read the journal's policy on disclosure of potential conflicts of interest. The authors declare that there are no conflicts of interest.

References

1. Choudhuri S, Klaassen CD. Structure, function, expression, genomic organization, and single nucleotide polymorphisms of human ABCB1 (MDR1), ABCC (MRP), and ABCG2 (BCRP) efflux transporters. Int J Toxicol 2006; 25(4):231-59.

2. Stieger B, Meier PJ. Pharmacogenetics of drug transporters in the enterohepatic circulation. Pharmacogenomics 2011; 12(5):611-31.

3. Delmas PD, Bjarnason NH, Mitlak BH, et al. Effects of raloxifene on bone mineral density, serum cholesterol concentrations, and uterine endometrium in postmenopausal women. N Engl J Med 1997; 337(23):1641-7.

4. Johnston CC, Jr., Bjarnason NH, Cohen FJ, et al. Long-term effects of raloxifene on bone mineral density, bone turnover, and serum lipid levels in early postmenopausal women: three-year data from 2 double-blind, randomized, placebo-controlled trials. Arch Intern Med 2000; 160(22):3444-50.

5. Ettinger B, Black DM, Mitlak BH, et al. Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year

randomized clinical trial. Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators. JAMA 1999; 282(7):637-45.

6. Siris ES, Harris ST, Eastell R, et al. Skeletal effects of raloxifene after 8 years: results from the continuing outcomes relevant to Evista (CORE) study. J Bone Miner Res 2005; 20(9):1514-24.

7. Hochner-Celnikier D. Pharmacokinetics of raloxifene and its clinical application. Eur J Obstet Gynecol Reprod Biol 1999; 85(1):23-9.

8. Jeong EJ, Liu Y, Lin H, Hu M. Species- and disposition model-dependent metabolism of raloxifene in gut and liver: role of UGT1A10. Drug Metab Dispos 2005; 33(6):785-94.

9. U.S.FDA. NDA-020-815 Rockville MD; 1999; Available from: http://www.fda.gov/cder/foi/nda/99/20815S3_Evista.htm (Last accessed: 10February 2009).

10. Dodge JA, Lugar CW, Cho S, et al. Evaluation of the major metabolites of raloxifene as modulators of tissue selectivity. J Steroid Biochem Mol Biol 1997; 61(1-2):97-106.

11. Chang JH, Kochansky CJ, Shou M. The role of P-glycoprotein in the bioactivation of raloxifene. Drug Metab Dispos 2006; 34(12):2073-8.

12. Jeong EJ, Lin H, Hu M. Disposition mechanisms of raloxifene in the human intestinal Caco-2 model. J Pharmacol Exp Ther 2004; 310(1):376-85.

13. Hoffmeyer S, Burk O, von Richter O, et al. Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity in vivo. Proc Natl Acad Sci U S A 2000; 97(7):3473-8.

14. Hemauer SJ, Nanovskaya TN, Abdel-Rahman SZ, Patrikeeva SL, Hankins GD, Ahmed MS. Modulation of human placental P-glycoprotein expression and activity by MDR1 gene polymorphisms. Biochem Pharmacol 2010; 79(6):921-5.

15. Foote CJ, Greer W, Kiberd BA, et al. MDR1 C3435T polymorphisms correlate with cyclosporine levels in de novo renal recipients. Transplant Proc 2006; 38(9):2847-9.

16. Basic S, Hajnsek S, Bozina N, et al. The influence of C3435T polymorphism of ABCB1 gene on penetration of phenobarbital across the blood-brain barrier in patients with generalized epilepsy. Seizure 2008; 17(6):524-30.

17. Saitoh A, Singh KK, Powell CA, et al. An MDR1-3435 variant is associated with higher plasma nelfinavir levels and more rapid virologic response in HIV-1 infected children. AIDS 2005; 19(4):371-80.

18. Naesens M, Kuypers DR, Verbeke K, Vanrenterghem Y. Multidrug resistance protein 2 genetic polymorphisms influence mycophenolic acid exposure in renal allograft recipients. Transplantation 2006; 82(8):1074-84.

19. Laechelt S, Turrini E, Ruehmkorf A, Siegmund W, Cascorbi I, Haenisch S. Impact of ABCC2 haplotypes on transcriptional and posttranscriptional gene regulation and function. Pharmacogenomics J 2011; 11(1):25-34.

20. Briggs B, Baker, PJ ,Belvo, MD, Black, TD, Getman, BG, Kemp, CAJ, Muth, WL, Perun, TJ, Strobel Jr, RJ, Paschal, JW and Zmijewski, MJ. Microbial process for preparation of glucuronides of raloxifene. Journal of Industrial Microbiology & Biotechnology 1999; 23:194–7.

21. Trdan T, Roskar R, Trontelj J, Ravnikar M, Mrhar A. Determination of raloxifene and its glucuronides in human urine by liquid chromatography-tandem mass spectrometry assay. J Chromatogr B Analyt Technol Biomed Life Sci 2011; 879(23):2323-31.

22. Berginc K, Zakelj S, Kristl A. In vitro interactions between aged garlic extract and drugs used for the treatment of cardiovascular and diabetic patients. Eur J Nutr 2010; 49(6):373-84.

23. Noe J, Stieger B, Meier PJ. Functional expression of the canalicular bile salt export pump of human liver. Gastroenterology 2002; 123(5):1659-66.

24. de Waart DR, Paulusma CC, Kunne C, Oude Elferink RP. Multidrug resistance associated protein 2 mediates transport of prostaglandin E2. Liver Int 2006; 26(3):362-8.

25. Gerloff T, Stieger B, Hagenbuch B, et al. The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver. J Biol Chem 1998; 273(16):10046-50.

26. Trontelj J, Bogataj M, Marc J, Mrhar A. Development and validation of a liquid chromatography-tandem mass spectrometry assay for determination of raloxifene and its metabolites in human plasma. J Chromatogr B Analyt Technol Biomed Life Sci 2007; 855(2):220-7.

27. Schmidt-Gayk H, Spanuth E, Kotting J, et al. Performance evaluation of automated assays for beta-CrossLaps, N-MID-Osteocalcin and intact parathyroid hormone (BIOROSE Multicenter Study). Clin Chem Lab Med 2004; 42(1):90-5.

28. Dahan A, Amidon GL. Small intestinal efflux mediated by MRP2 and BCRP shifts sulfasalazine intestinal permeability from high to low, enabling its colonic targeting. Am J Physiol Gastrointest Liver Physiol 2009; 297(2):G371-7.

29. Luders AK, Saborowski R, Bickmeyer U. Inhibition of multidrug/xenobiotic resistance transporter by MK571 improves dye (Fura 2) accumulation in crustacean tissues from lobster, shrimp, and isopod. Comp Biochem Physiol C Toxicol Pharmacol 2009; 150(3):368-71.

30. Hegedus C, Szakacs G, Homolya L, et al. Ins and outs of the ABCG2 multidrug transporter: an update on in vitro functional assays. Adv Drug Deliv Rev 2009; 61(1):47-56.

31. Jeannesson E, Albertini L, Siest G, et al. Determination of ABCB1 polymorphisms and haplotypes frequencies in a French population. Fundam Clin Pharmacol 2007; 21(4):411-8.

32. Keskitalo JE, Zolk O, Fromm MF, Kurkinen KJ, Neuvonen PJ, Niemi M. ABCG2 polymorphism markedly affects the pharmacokinetics of atorvastatin and rosuvastatin. Clin Pharmacol Ther 2009; 86(2):197-203.

33. Aungst BJ, Nguyen NH, Bulgarelli JP, Oates-Lenz K. The influence of donor and reservoir additives on Caco-2 permeability and secretory transport of HIV protease inhibitors and other lipophilic compounds. Pharm Res 2000; 17(10):1175-80.

34. Faassen F, Kelder J, Lenders J, Onderwater R, Vromans H. Physicochemical properties and transport of steroids across Caco-2 cells. Pharm Res 2003; 20(2):177-86.

Legends for figures

Fig. 1: Inhibitory effects of raloxifene, raloxifene-6-β-glucuronide (M1), raloxifene-4'-β-glucuronide (M2), raloxifene-6,4'-diglucuronide (M3) on active uptake of estradiol-17β-glucuronide (E17βG) into MRP2 expressing membrane vesicles. Each column represents the mean (SE) (n=4) and (*) indicates a significant difference from the control ($p < 0.05$).

Figure1

Click here to download high resolution image

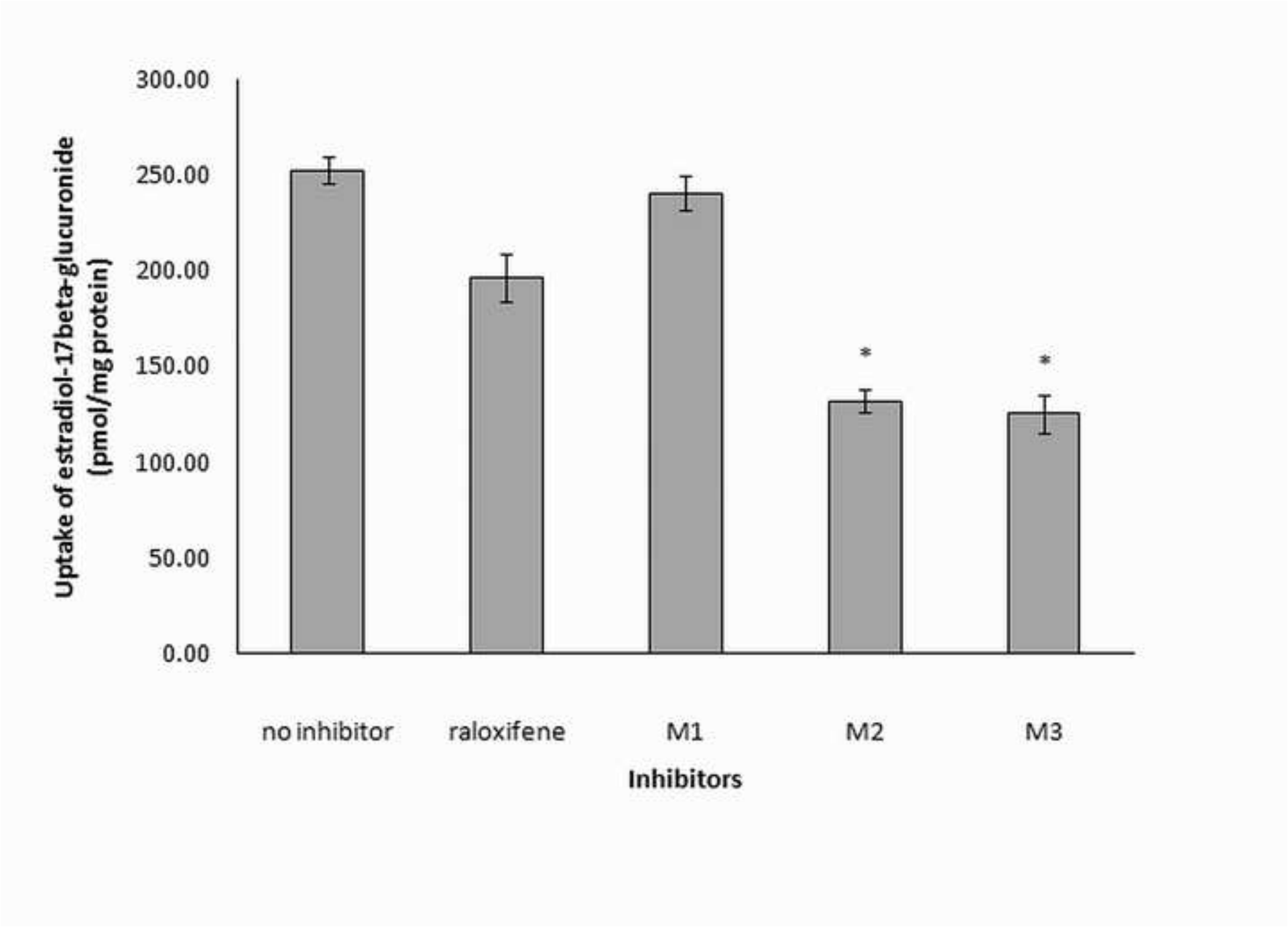

Table1

Table 1

Passive permeability of raloxifene species.

	P_{app} (*10^{-7} cm/s)
RAL	27.0 ± 8.0
M1	1.4 ± 0.17
M2	0.88 ± 0.15
M3	0.51 ± 0.007

The values in table are presented as mean (±standard deviation).

RAL, raloxifene; M1, raloxifene-6-β-glucuronide; M2, raloxifene-4'- β-glucuronide; M3, raloxifene-6,4'- diglucuronide

Table 2

The influence of specific inhibitors of transporters on efflux ratio of raloxifene

Caco-2 permeability (*10^{-6} cm/s)				
Inbibitor	**AP-BL**	**BL-AP**	**ER**	**S/NS**
reference	7.9 ± 1.1	11.7 ± 1.6	1.48 ± 0.29	
Ver [100μM]	13.1 ± 1.0	6.2 ± 0.9	0.47 ± 0.08	S
MK [25μM]	9.2 ± 1.8	10.3 ± 2.7	1.12 ± 0.37	NS
FumC [10μM]	8.2 ± 1.1	8.4 ± 1.7	1.02 ± 0.25	NS

The ER values represent the ratio between BL–AP (basolateral-apical) and AP–BL(apical-basolateral) permeabilities of raloxifene. The values in table are presented as mean (±standard deviation).

S/NS statistical analysis of ER (efflux ratio) change comparing with reference, S significant ($p<0.05$), NS not significant ($p>0.05$). Ver, verapamil; MK, MK571; FumC, fumitremorgin C.

Table 3A

Quantitative ATPase assay results obtained in baseline, activation and inhibition experiments

Averages of released Pi at baseline / activation / inhibition experiments [nmol/mg(protein)/min]					
	Pgp	**MRP1**	**MRP2**	**MRP3**	**BCRP**
RAL	11.5/34.4/7.3	9.9/14.9/0.9	5.9/10.9/-1.4	3.7/15.3/2.5	2.8/12.3/-19.9
M1	20.3/47.2/14.5	13.2/17.1/-0.1	7.9/18.1/-1.5	13.5/22.3/6.8	5.0/25.7/-15.0
M2	14.8/43.5/7.8	10.9/14.5/-1.4	7.6/20.2/0.2	10.2/20.0/8.4	5.2/15.6/-20.3
M3	10.9/48.5/11.5	8.1/18.6/0.7	7.5/16.7/-0.9	4.4/12.4/2.4	8.6/31.4/-15.7
Estimated IC50 values* [µM]					
RAL	6	5	2	12	6
M1	10	1	n.d.**	10	40
M2	6	4	1.5	8	0.25
M3	0.75	2	nd**	0.5	3

*** IC50 values were estimated from ATP dependent transport curves versus raloxifene species concentrations (figures not shown).**

**** the slope of the concentration-ATP-ase inhibition curve was not significantly different from 0**

Table3b

Table 3 B

Presentation of ATPase assay results

Activation experiment					
	Pgp	MRP1	MRP2	MRP3	BCRP
RAL	+	n.e.	n.e.	+	n.e.
M1	n.e.	n.e.	n.e.	+	n.e.
M2	n.e.	n.e.	n.e.	+	n.e.
M3	+	+	n.e.	n.e.	n.e.
Inhibition experiment					
	Pgp	MRP1	MRP2	MRP3	BCRP
RAL	--	--	--	-	--
M1	-	--	n.e.	-	-
M2	--	--	--	--	--
M3	-	-	-	-	-

\+ weak activation

++ strong activation (was not detected)

\- weak inhibition

-- strong inhibition

n.e….no effect

RAL, raloxifene; M1, raloxifene-6-β-glucuronide; M2, raloxifene-4'-β-glucuronide; M3, raloxifene-6,4'-diglucuronide

Table 4

Genotype frequencies of *ABCB1* and *ABCC2* polymorphisms

Genetic variant	Genotype	Number of patients	Frequency (%)
ABCB1 **c.3435C>T**	CC	17	29.8
	CT	24	42.1
	TT	16	28.1
ABCC2 **c.3972C>T**	CC	26	45.6
	CT	21	36.8
	TT	10	17.5

Table 5

Summary of concentration of raloxifene species according to *ABCB1* c.3435C>T and *ABCC2* c.3972C>T polymorphisms

	***ABCB1* c.3435C>T**				***ABCC2* c.3972C>T**			
	CC	**CT**	**TT**	**p value (ANOVA)**	**CC**	**CT**	**TT**	**p value (ANOVA)**
c (M1) (nmol/L)	49 (10)	83 (14)	79 (23)	0.170	58 (9)	94 (20)	50 (11)	0.282
c (M2) (nmol/L)	199 (42)	402 (77)	347 (90)	0.090	270 (53)	424 (86)	206 (48)	0.344
c (M3) (nmol/L)	301 (59)	582 (107)	675 (270)	0.173	333 (53)	711 (202)	529 (197)	0.192
c (RAL) (nmol/L)	2.8 (0.4)	4.3 (0.7)	3.6 (0.9)	0.377	3.6 (0.5)	3.4 (0.6)	4.0 (1.4)	0.976
c (TR) (nmol/L)	552 (99)	1071 (171)	1105 (360)	0.111	665 (105)	1232 (279)	789 (251)	0.232

The values of concentrations are presented as means (standard errors).

RAL, raloxifene; M1, raloxifene-6-β-glucuronide; M2, raloxifene-4'-β–glucuronide; M3, raloxifene-6,4'-di–glucuronide; TR, total raloxifene

Table 6

Percentage change of bone mineral density of total hip according to the presence of *ABCB1* c.3435C>T polymorphism after 12 months of raloxifene treatment in 53 postmenopausal women.

	***ABCB1* c.3435C>T**				***ABCC2* c.3972C>T**			
	CC	**CT**	**TT**	**p value (ANOVA)**	**CC**	**CT**	**TT**	**p value (ANOVA)**
Δ BMD-HIP (%)	-2.1 (2.5)	-0.5 (0.9)	6.5 (3.0)	0.016[a]	0.03 (2.4)	2.2 (1.8)	-2.1 (1.7)	0.809
Δ BMD-LS (%)	0.6 (1.1)	2.5 (1.0)	1.3 (1.4)	0.523	1.4 (1.1)	1.7 (0.9)	2.0 (1.5)	0.608
Δ BMD-FN (%)	0.2 (1.3)	2.3 (0.9)	1.3 (1.7)	0.589	2.3 (0.9)	0.4 (1.0)	0.8 (2.5)	0.269
Δ BUA (%)	-1.0 (2.2)	2.3 (1.7)	5.9 (2.4)	0.399	1.5 (1.6)	1.4 (2.0)	5.9 (3.6)	0.431
Δ SOS (%)	-0.03 (0.15)	-0.09 (0.19)	0.12 (0.11)	0.230	-0.02 (0.16)	0.09 (0.17)	-0.22 (0.15)	0.660
Δ QUI (%)	-0.2 (1.5)	-0.2 (2.0)	2.8 (1.1)	0.283	0.3 (1.4)	1.8 (2.1)	-0.05 (1.8)	0.840
Δ BALP (%)	-24.3 (6.3)	-16.9 (8.3)	-29.9 (3.9)	0.434	-29.0 (4.8)	-13.6 (9.2)	-22.5 (8.2)	0.269
Δ OC (%)	-24.1 (5.3)	-28.2 (4.5)	-22.6 (4.2)	0.770	-23.5 (3.9)	-27.5 (5.3)	-27.2 (5.1)	0.635
Δ CTX (%)	-22.9 (7.5)	-32.0 (5.8)	-18.9 (8.1)	0.601	-18.1 (6.2)	-37.4 (4.9)	-23.5 (10.5)	0.315

The values are presented as means (standard errors). [a]p(1,3)=0.024